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CONTRACT NO: DAMD17-87-C-7187

TITLE: MOLECULAR BIOLOGY OF STLV-III AND HTLV-IV

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REPORT DATE: August 10, 1989

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Dana-Farber Cancer Institute, Laboratory of Biochemistry Pharmacology; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 44 Binney Street, Boston, MA 02115	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-87-C-7187
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21702-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63105A; PROJECT NO. 3M2 63105H29; TASK NO. AC; WORK UNIT ACCESSION NO. 072

11. TITLE (Include Security Classification)

Molecular Biology of STLV-III and HTLV-IV

12. PERSONAL AUTHOR(S)

Joseph Sodroski, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 7/1/88 TO 6/30/89	1989 August 10	17

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	13	

18. SUBJECT TERMS (~~Continue on reverse if necessary and identify by block number~~)

Virology; Molecular Biology; Biochemistry; Human Immunodeficiency Virus; ~~HTLV-IV; STLV-III~~; Simain AIDS; ~~RAI~~

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Molecular characterization of the $SIV_{mac}251$ clone that exhibits *in vitro* replication competence, but poor *in vivo* replication ability was initiated by complete nucleotide sequence determination. Defects in the *vpr*, *nef* and *env* genes were noted and will be the focus of future efforts to restore *in vivo* replication competence of this cloned virus.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Virginia M. Miller	22b. TELEPHONE (Include Area Code): (301) 663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

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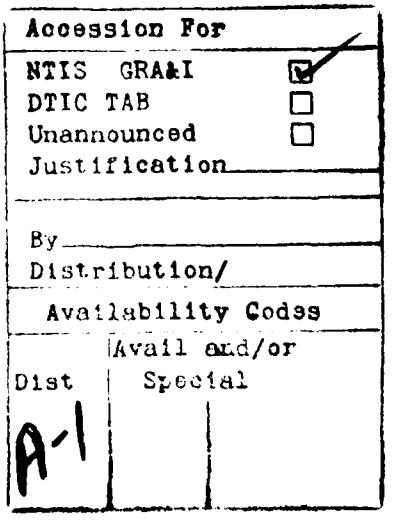


Addition information for Methods section.

The pFLB10 proviral DNA was sequenced according to the following method. Oligonucleotide primers were prepared based on consensus sequences for available SIV isolates provided in the Los Alamos data bank. Approximately 85% of these primers were found to yield readable sequence information from the pFLB10 provirus. An additional set of primers was prepared based on the sequence of cDNA clones derived from SIVmac 251 - infected cells. The latter primers were found to prime readable sequencing reactions using the pFLB10 provirus in nearly 100% of the cases. In total, sequencing reactions with approximately 45 different primers were used to complete the sequence of the entire pFLB10 provirus.

Sequencing reactions were carried out according to standard protocols based upon the method of Sanger et al. Briefly, template DNA was prepared by treatment of the pFLB10 plasmid with alkali to denature the DNA, followed by strand separation on denaturing acrylamide gels. The primer was annealed to the single-stranded template DNA by heating to 65^{0}C then slowly cooling the reaction to 35^{0}C. The molar ratio of primer:template for most reactions ranged from 1:1 to 3:1. The annealed primer:template was mixed with the 6mM DTT, labeling mix and sequenase enzyme (Sequenase Kit, USB Corporation) and [x-^{35}S] dATP, then incubated at room temperature for 2-5 minutes. The reactions were terminated by addition of ddATP, ddGTP, ddTTP and ddCTP, incubation at 37^{0}C for 3-5 minutes, and addition of stop solution (Sequenase Kit, USB Corporation). Samples were loaded on simultaneous 4% and 8% acrylamide sequencing gels, or in some cases, on 6% gradient gels. Each sequencing reaction typically yielded from 400 to 550 base pairs of information. Each segment of the genome was sequenced using at least two sets of non-overlapping primers.

Molecular Analysis of an Infectious $SIV_{mac}251$ Proviral Clone

Abstract

Molecular characterization of the $SIV_{mac}251$ proviral clone pFLB-10 was carried out. This $SIV_{mac}251$ isolate exhibits in vitro replication competence, but replicates poorly in vivo and, as a consequence is not pathogenic. Defects in the vpr, nef and env genes were noted that may be responsible for attenuations in in vivo replicative ability.

Introduction

The pFLB-10 provirus was derived from a bacteriophage library made from human T cells infected with $SIV_{mac}251$. The pFLB-10 provirus generates infectious SIV upon DEAE-dextran transfection of HUT78 and CEMX174 lymphocytes. Virus stocks of greater than $5x10^5$ reverse transcriptase units per milliliter were used for inoculation of rhesus macaques and cynomolgus monkeys. None of the animals inoculated with the FLB-10 virus has to date demonstrated a persistent viremia or induction of disease, whereas control animals inoculated the uncloned $SIV_{mac}251$ virus have developed AIDS-like illness in the same time period. The studies in animals are of one year duration and are ongoing. We conclude that changes in the molecularly cloned FLB-10 virus compromise its ability to allow efficient replication in vivo. Here we report the progress made in molecular analysis of this deficiency in in vivo replication/pathogenesis.

Methods

To understand the molecular characteristics of the FLB-10 virus that might be relevant to the observed decrease in in vivo replication, the infectious provirus was completely sequenced using the Sanger dideoxy technique (1). Using chemically synthesized oligonucleotides as primers and increasing the readable sequence information by using gradient urea gels and ^{35}S label, the nucleotide sequence could be obtained using a minimal number of individual priming reactions.

Results and Discussion

The sequence of the 3' half of the FLB-10 provirus is shown in appendix 1 and is summarized in figure 1. The nucleotide sequence of the FLB-10 clone is generally similar to that of other $SIV_{mac}251$ isolates and is more similar to those isolates than to SIV's derived from mangabeys or African green monkeys. The major open reading frames in the FLB-10 provirus are as follows:

1. vpx

The vpx protein is not necessary for SIV replication, but appears to stimulate virus replication through an unknown mechanism. The vpx open reading frame is present and a potential initiator methionine is evident at the 5' end of the reading frame. The FLB-10 vpx open reading frame could encode a protein of 113 amino acids, with a proline-rich segment near the carboxyl terminus. The vpx of FLB-10 differs from the consensus SIV sequence at one residue (62) M $\longrightarrow$ K.

2. vpr

The vpr protein is not necessary for HIV-1 gene expression, but can stimulate HIV-1 replication through its effect as a promiscuous trans-activator of gene expression. Most SIV_{mac} isolates have an open reading frame for vpr, with heterogeneity at the 3' end. The BK28 isolate encodes a 98 amino acid vpr product, while MM142 encodes a 102 amino acid vpr protein. The vpr open reading frame of the FLB-10 provirus has obviously lost the potential to encode a protein, since the methionine likely to be utilized for initiation, based on sequence similarity to other SIV_{mac} provirus clones, is followed within eleven codons by a stop codon. Following the stop codon, the vpr open reading frame continues with strong sequence similarity to the vpr sequence of other SIV isolates, so it is likely that correction of the single stop codon will result in a full-length vpr product. This potential vpr product would be 98 amino acids long, similar in size to functional vpr proteins observed in the HIV-1 system. In addition, there are two vpr changes in FLB-10 differing from the consensus SIV sequence (47) I $\longrightarrow$ M and (77) C $\longrightarrow$ S.

3. tat and rev

The FLB-10 tat and rev open reading frames are intact, as would be expected for an infectious proviral clone. Tat is a positive trans-activator of viral gene expression, while rev is a post-transcriptional regulator of structural protein expression. The tat gene of FLB-10 differs from consensus by only two changes (27) A $\longrightarrow$ R and (75) S $\longrightarrow$ C, while the FLB-10 rev gene demonstrates no changes from the SIV consensus sequence.

4. env

The FLB-10 envelope glycoproteins are similar to other SIV_{mac} isolates in that a premature stop codon exists in the transmembrane glycoprotein. It appears that either one of a pair of CAG residues is converted to a TAG amber codon in various SIV_{mac} isolates passaged in human cell lines. The FLB-10 clone has a TAG CAG sequence, similar to that seen in the $SIV_{mac}251$ but unlike the other SIV_{mac} isolates. In addition, the FLB-10 has a second premature stop codon that would result in a deletion of 18 carboxy-terminal amino acids.

5. nef

Although premature stop codons in HIV-1 nef genes are common, most SIV molecular clones do not have obvious premature nef stop codons. Heterogeneity occurs in the carboxyl terminus of nef in different SIV isolates (from 248-299 amino acid residues). The FLB-10 nef gene, which otherwise could encode a 263 residue protein, contains at least two defects that render it non-functional. First, the nef initiator methionine codon (ATG) has been mutated to an ATA, encoding isoleucine. The next residue, a glycine, which is important for myristillic acid addition, has been preserved in the FLB-10 provirus. Second, a premature stop codon results in only a 94 amino acid product, even if the initiator methionine were present. This result explains the observation that deletions of the FLB-10 provirus in the nef region did not result in phenotypic differences in viral replication rate or in cytopathic effect.

In summary, the molecular changes that might in part account for the attenuated in vivo replication rate of an $SIV_{mac}251$ infectious virus include changes in the vpr and nef genes as well as truncation of the transmembrane envelope glycoprotein.

Plans for the next year include mutagenesis of the FLB-10 provirus to examine the effects on in vitro and in vivo replication of restoring some of the mutated vpr, env, and nef sequences. The reading frames that are intact, like vpx and vif, will be deleted to examine the resulting phenotype.

References

1. Sanger F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. PNAS 74: 5463.

Figure 1. Schematic diagram of the FLB-10 3' half of the provirus. The vpx, vpr, tat, rev, env and nef genes of the FLB-10 SIV_{mac} 251 isolate are shown. The X's represent changes in the FLB-10 sequence that render the open reading frame products either unable to be synthesized or prematurely terminated. The dark boxes represent the two coding exons of the functional tat and rev genes of FLB-10.

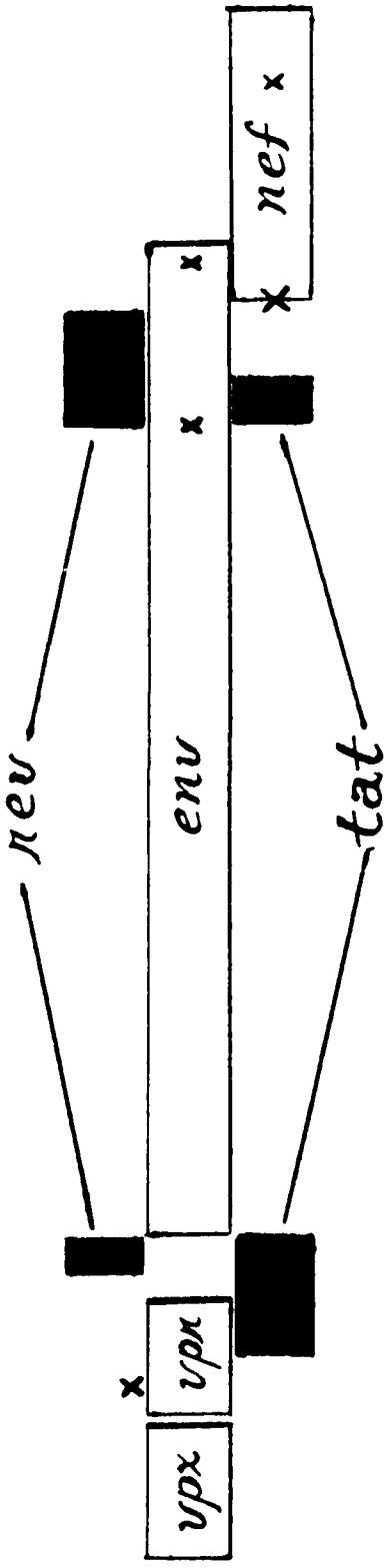
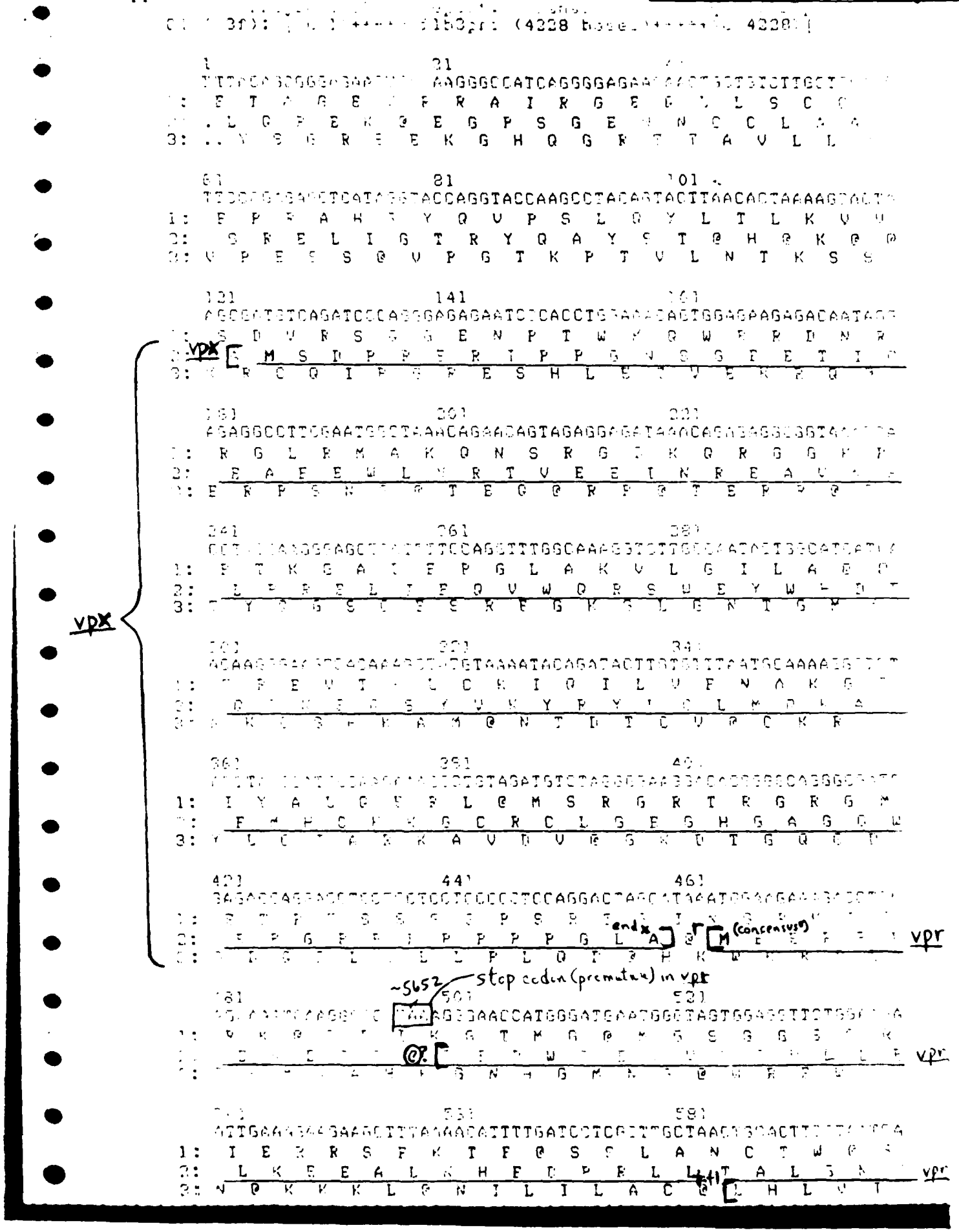


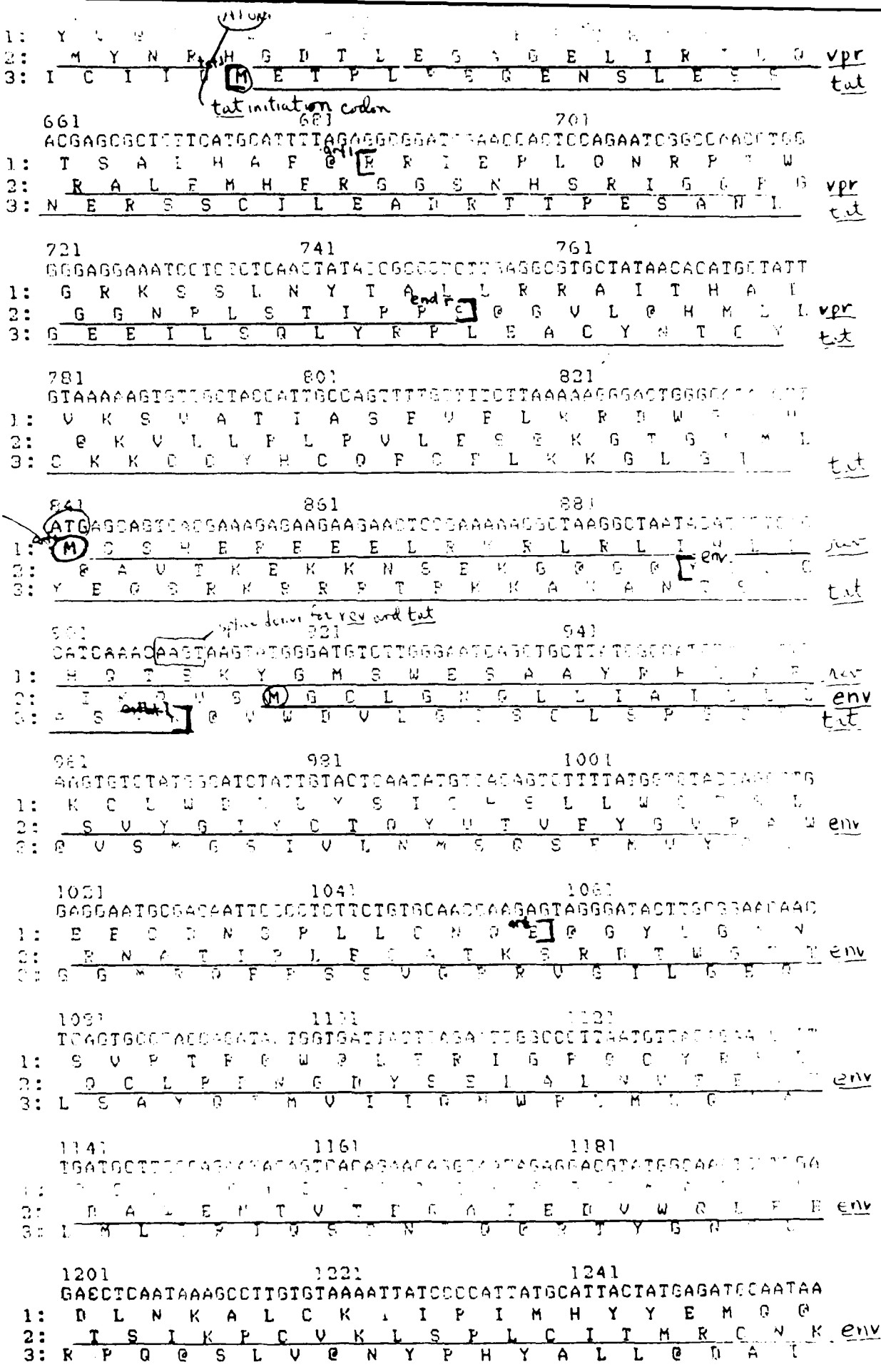
Figure 1.

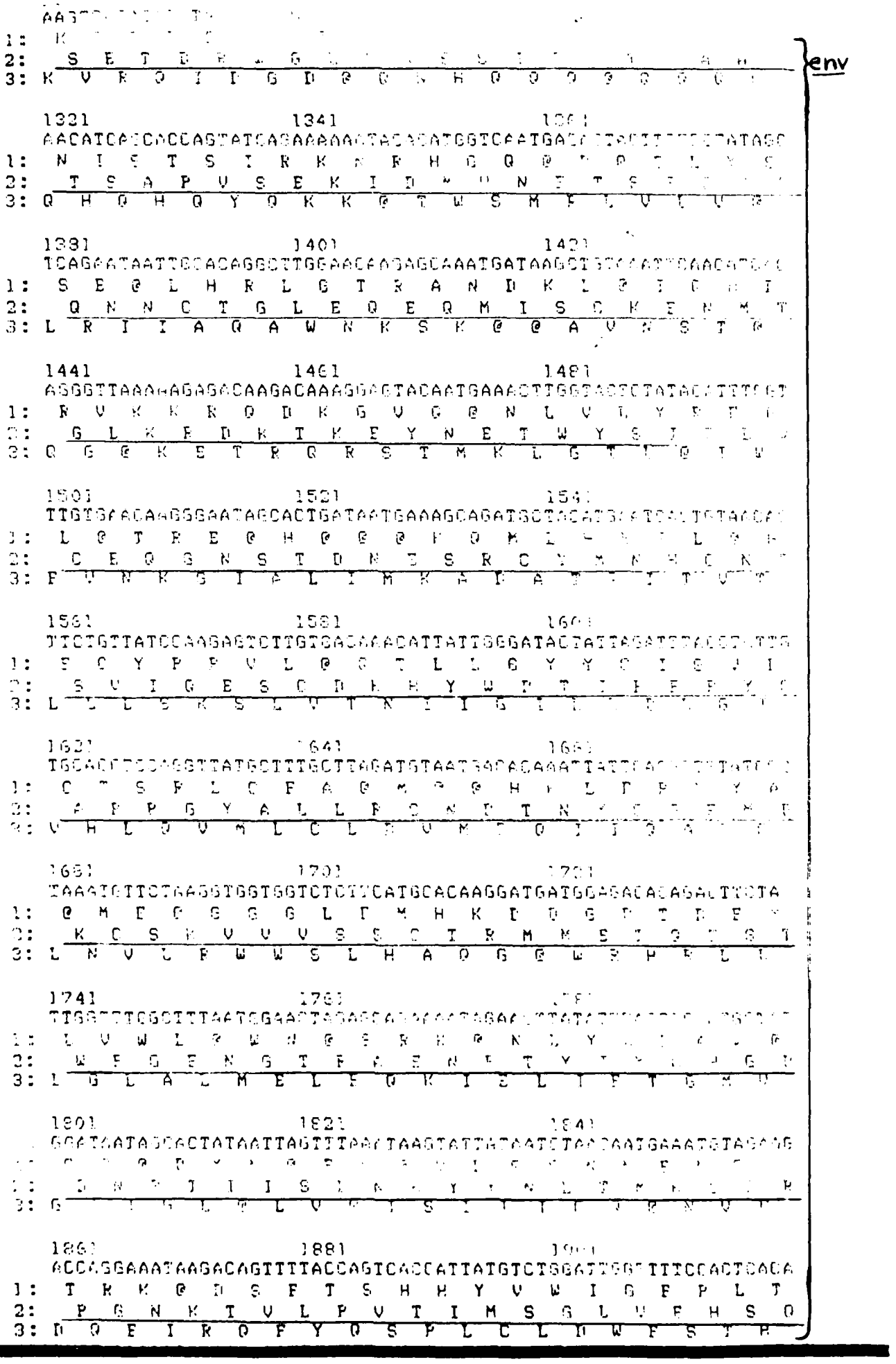
Appendix 1. Complete nucleotide sequence of the 3' half of the FLB-10 provirus, with open reading frames underlined.

Appendix 1.

SIVmac251 HLB-10 3' segment




tat initiation codon
initiation codon rev
splice donor for rev and tat
vpr
tat
rev
env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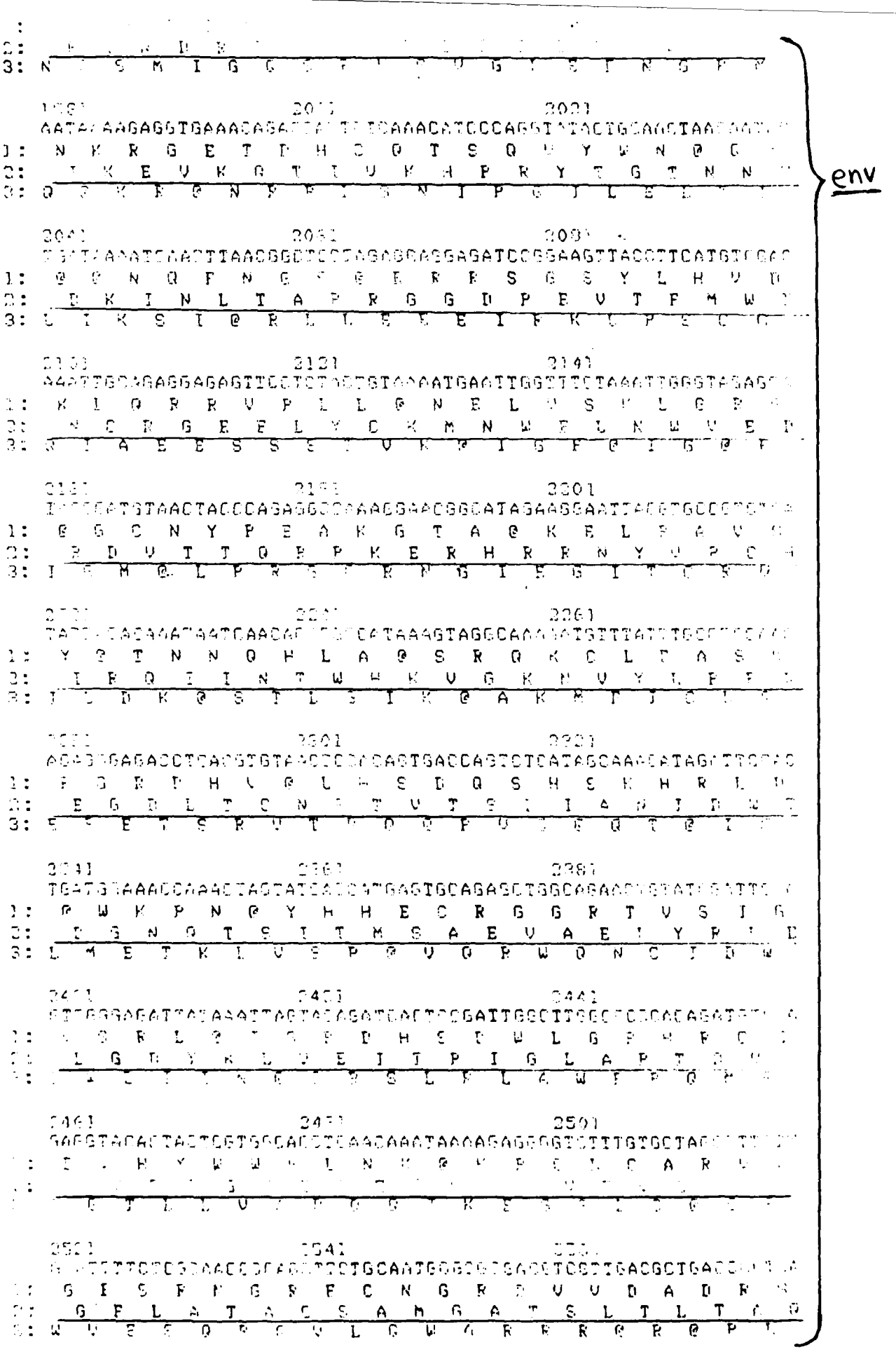

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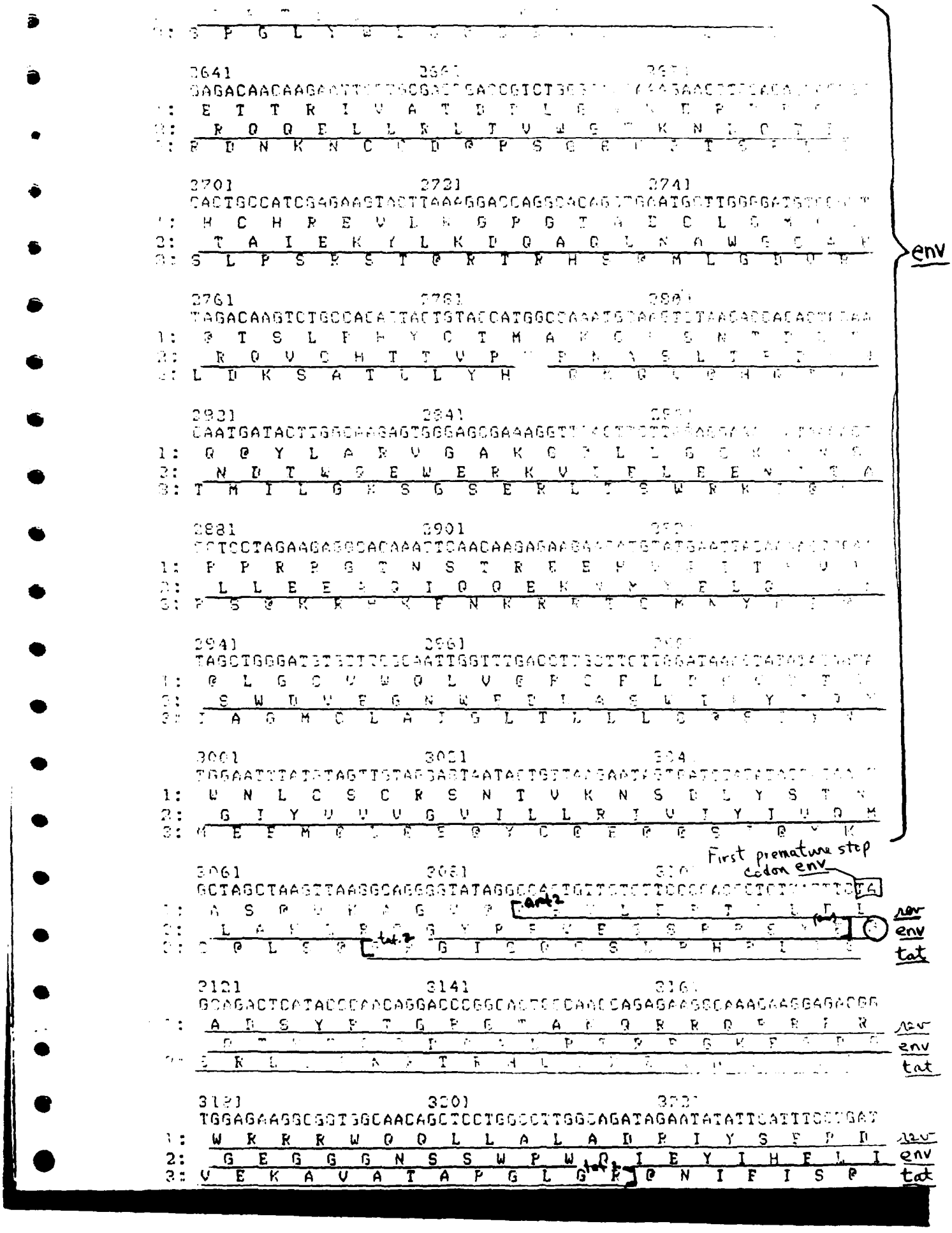
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rev
env
tat
rev
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tat
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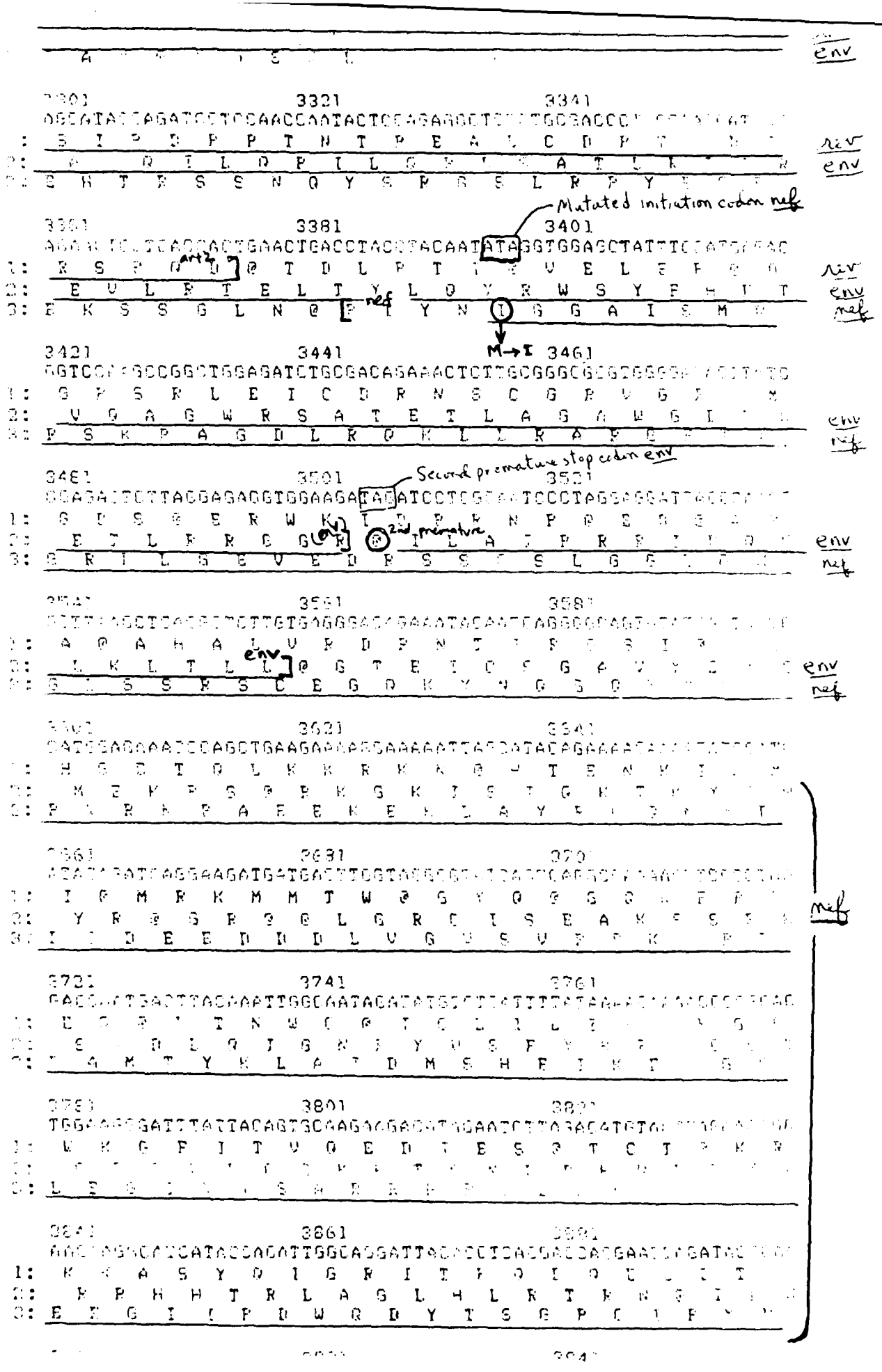
tat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3321
3341
rev
env
Mutated initiation codon nef
3361
3381
3401
rev
env
nef
M→I
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3461
env
nef
Second premature stop codon env
3481
3501
3521
2nd premature
env
nef
3541
3561
3581
env
nef
3601
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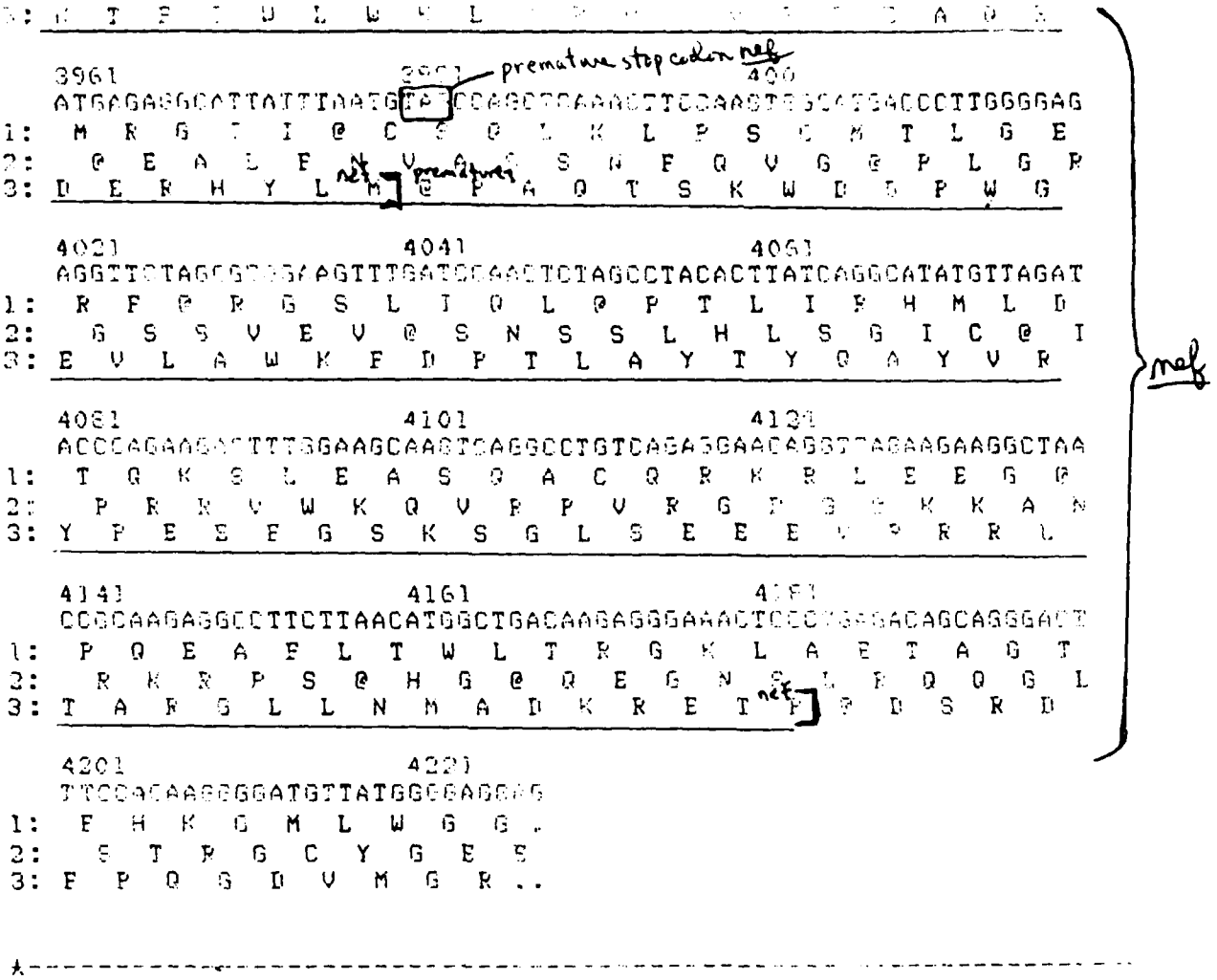
premature stop codon
nef
400
nef
nef
nef